Report for 2004MN65B: The Effects of Long-Term Low-Level Antibiotic Exposure on the Development of Antibiotic Resistance

- Other Publications:
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Report Follows

The Effects of Long-Term Low-Level Antibiotic Exposure on the Development of Antibiotic Resistance

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Summary
Antibacterial compounds have been detected in the environment at low, subtherapeutic levels. Here, we examined whether the presence of antibacterials at these levels would lead to an increase in antibacterial resistance among exposed bacteria. Three chemostats were operated with identical enrichment cultures from Mississippi River water. One chemostat contained no antibacterials, a second contained four antibacterials (sulfamethoxazole, norfloxacin, trimethoprim, and tylosin) as a mixture at very low concentrations (≤ 1 μg/L each), and a third contained norfloxacin at a 1 μg/L concentration. Enumeration of the proportion of bacteria from each chemostat exhibiting antibiotic resistance was performed approximately weekly using heterotrophic plate counts on nutrient media supplemented with elevated levels of each antibiotic. Ability of the bacteria to grow on liquid media supplemented with each antibiotic was also tested periodically. Polymerase chain reaction followed by denaturing gradient gel electrophoresis (PCR-DGGE) was used to track changes in the community structure over time. No significant differences were observed for the bacterial populations from the three chemostats, which suggests that very low antibiotic concentrations may not select for antibiotic resistant bacteria in the environment.

Introduction
The presence of pharmaceutical and personal care products (PPCPs) in natural waters has gained attention due to recent reports (*1-5*) including a national reconnaissance of United States streams conducted by the U.S. Geological Survey in which pharmaceuticals were detected at 80% of the sites sampled (*6*). The widespread occurrence of antibacterial compounds is especially alarming for several reasons. First, the presence of antibiotics in natural waters may lead to the proliferation of antibiotic resistance among exposed microorganisms, and this resistance may be transferable to human pathogens (*7-10*). Alternatively, if present at high enough concentrations, antibacterial compounds may adversely affect native microbial communities.

Antibacterial compounds have been detected thus far in natural waters at concentrations far below therapeutic levels. While there is a concern that long-term exposure at these low levels may result in an increase in antibiotic resistance among environmental bacteria, this relationship has not yet been conclusively established through studies in environmental systems (*11-13*). This project was designed to explicitly study the suspected link between low-level antibacterial exposure and proliferation of resistant bacteria by exposing environmental bacteria to

subtherapeutic levels of antibacterial compounds and testing for increases in resistance. To circumvent the difficulty in establishing causation of resistance in field studies, laboratory studies were performed using chemostats (Figure 1) in which the presence or absence of long-term constant exposure to subtherapeutic antibacterial concentrations was the only system variable.

Methods

Chemostat operation

A sample of Mississippi River water was collected in Minneapolis, MN, aliquoted into 2 mL samples, and preserved in 15% glycerol stocks at -70 °C to provide bacteria for seeding the chemostats. For each chemostat, an initial inoculum was prepared by adding 1 mL of glycerol stock to 600 mL of PYT80 liquid medium (80 mg peptone, 80 mg yeast extract, 80 mg tryptone per liter of 10 mM Tris buffer). Chemostats were inoculated after 3 days of growth on the PYT80. Table 1 summarizes the operating conditions of the three chemostats. The nutrient medium for all chemostats was PYT80. The nutrient medium for chemostat **2** was supplemented with four antibacterial compounds: tylosin (a macrolide), trimethoprim, sulfamethoxazole (a sulfa drug) and norfloxacin (a fluoroquinolone). Concentrations were similar to what has been observed in the environment. The nutrient medium for chemostat **3** contained only norfloxacin at a slightly higher concentration than in **2**. Prior to beginning the chemostat experiments, tests to determine the potential significance of photolysis, hydrolysis, and autoclaving in the degradation of each antibacterial were performed. An HPLC equipped with a UV-absorbance detector was used to measure antibacterial concentrations. Limits of detection required that these tests be performed at higher concentrations than those used in the chemostats. Optical density at 600 nm (OD_{600}) and pH were monitored daily using the effluent from each chemostat.

Table 1: Relevant parameters for the three chemostats in the study.

Chemostat	*Dates Operated (all in 2004)*	*Total Days*	*Antibiotic Concentrations (μg/L)*	*Approximate Residence Time (hr)*
1	5/26 – 11/14	172	none	24
2	6/8 – 9/7	91	Tylosin: 1 Trimethoprim: 0.3 Sulfamethoxazole: 0.5 Norfloxacin: 0.1875	24
3	9/29 – 11/12	44	Norfloxacin: 1	24

Resistance testing: plate counts

Five types of plates were prepared (all with PYT80 solid medium); one type contained no supplement, and four other types contained one of the following: 200 mg/L tylosin, 20 mg/L trimethoprim, 20 mg/L sulfamethoxazole, or 20 mg/L norfloxacin. The number of bacteria able to grow on each of the antibacterial-supplemented plates was compared to the number able to grow on the plain PYT80 plates to determine the percentage of bacteria from each chemostat exhibiting antibacterial resistance. Plate counts were performed approximately every seven days for **1** and **2**; plate counts were not performed for **3**. Serial dilutions were used and counts were only performed for the dilutions (typically 10^{-4} to 10^{-7}) that resulted in 30-300 CFUs per plate after incubation at lab temperature for 2-3 days. All counts were performed in triplicate.

Resistance testing: liquid media
Iso-Sensitest broth (ISB) was prepared in pH 7 phosphate buffer with no supplement or with one of the four antibacterial compounds at the same concentrations as in the plate counts. 100 µL of the effluent from the chemostat was added to 10 mL of each of the five types of ISB medium in test tubes (in triplicate). Test tubes were incubated at lab temperature and rigorously shaken (200 rpm) for 24 hours. OD_{600} after growth on ISB supplemented with each antibacterial was compared to OD_{600} after growth on plain ISB. Growth tests on liquid media were performed approximately every one to two weeks for **1** from Day 41 onward, for **2** from Day 29 onward, and for **3** (norfloxacin-supplemented tubes only) throughout the duration of the chemostat's operation.

Community analysis
1.5 mL of effluent was collected daily from each chemostat; cells were pelleted, resuspended in 0.5 mL of lysis buffer (120 mM sodium phosphate, 5% SDS, pH 8), and preserved at -20 °C. After collection of samples for several months, DNA was extracted from the cells and polymerase chain reaction (PCR) was used to amplify 16S ribosomal RNA gene fragments. Denaturing gradient gel electrophoresis (DGGE) was performed to the PCR-amplified gene fragments to provide a fingerprint of bacterial community structure. Details of DNA extraction, PCR, and DGGE protocols are omitted here.

Results
One of the major benefits from this project was gaining an understanding of the techniques that will be required in the future to operate the chemostats consistently and under the desired conditions. OD_{600} and pH measurements were reasonably stable over time and no significant differences were observed among the chemostats. Testing showed that losses due to hydrolysis should not be significant for any of the four antibacterial compounds within the approximately two week time period that the compounds were present in the feed tank. Photolysis under laboratory lights was expected to lead to some loss for both norfloxacin and tylosin, so initial concentrations were raised slightly from what had been originally planned to adjust for this loss.

The biggest challenge proved to be avoiding contamination in the chemostats supplemented with antibacterials. Chemostats **2** and **3**, in addition to two short-lived chemostats not included in Table 1, were all terminated due to contamination of their feed tanks. Examination of the stability of the antibacterials under the autoclaving procedure used to prepare the feed media revealed that loss of norfloxacin (20%) and tylosin (43%) were much too large to allow inclusion of the antibacterial compounds in the media prior to autoclaving. Therefore, the compounds were added aseptically after autoclaving, and this proved challenging. Filter sterilization of antibacterial stock solutions helped, but this remains a future challenge for these experiments.

Plate counts revealed no measurable differences between resistance levels in chemostats **1** and **2** for sulfamethoxazole, trimethoprim, and tylosin, and no discernable trends in resistance levels over time were observed for either reactor. For norfloxacin, overall resistance levels were slightly higher in **2** (1 - 50%) than in **1** (0.1 – 11%) but variability was too high to determine that it was a significant difference. Again, no clear trends in resistance over time were observed.

Because variability was so high in the plate counts, the tests of resistance levels using liquid cultures were implemented. Liquid cultures, however, provide different information than the plate counts. Plate counts allow a count of overall resistance; in other words the total percentage that can grow over time is measured. With liquid cultures, if there are just a few faster-growing organisms, these can dominate the measurement. In addition, heterotrophic plate counts allow visual assessment of whether shifts in the phenotypes of the community are occurring over time, and this is not possible in the liquid culture tests. Therefore, variability is smaller but less information is obtained. Comparison of growth in liquid media revealed no differences among chemostats **1**, **2**, and **3** with the possible exception of differences in growth on sulfamethoxazole-supplemented media between **1** and **2**. Growth in the presence of sulfamethoxazole was consistently higher for bacteria from reactor **2**. The absence of data of this type from approximately the first month of operation makes it difficult to comment on trends in the data; further work should be done to determine if a higher resistance level (by this measure) did develop over time in chemostat **2**.

PCR-DGGE analysis showed some small changes in the community structure of each reactor over time. Figure 2 shows that over the first 48 days that **2** was operated there may have been some loss of diversity; some bands faded or disappeared over time. The changes were subtle, however, and the community was not obviously affected by the presence of low levels of antibiotics. Similar levels of change were observed for the other chemostats. Therefore, impacts of the antibacterial compounds on the bacteria at the levels studied appear to have been minimal by all three measures: plate counts, liquid media, and community analysis.

Future work

Further work is planned by Dr. Wammer upon arrival at the University of St. Thomas in Fall 2005 to build upon what was learned during the project period. Chemostats will be used to examine effects of antibacterial compounds at higher concentrations (closer to biologically relevant levels) on enrichment cultures from Mississippi River water to determine what concentrations are required for measurable effects on bacterial populations to be observed.

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Description of student training provided by project:

Name: Leslie J. Onan
Department: Civil Engineering, University of Minnesota
Degree being sought: B.S.

Name: Christian G. Klatt
Department: Civil Engineering, University of Minnesota
Degree earned: B.S. (chemical engineering)

Statement of related grants submitted or funded as a result of this project

Dr. Wammer has submitted a proposal for a new faculty award to the Camille and Henry Dreyfus Foundation and plans to be a co-investigator on a proposal to be submitted to the National Science Foundation in September 2005. Portions of both proposals request funding to study aspects of the problem addressed in this project on a more comprehensive scale, and it is believed that the preliminary results generated during this project will strengthen the proposals.

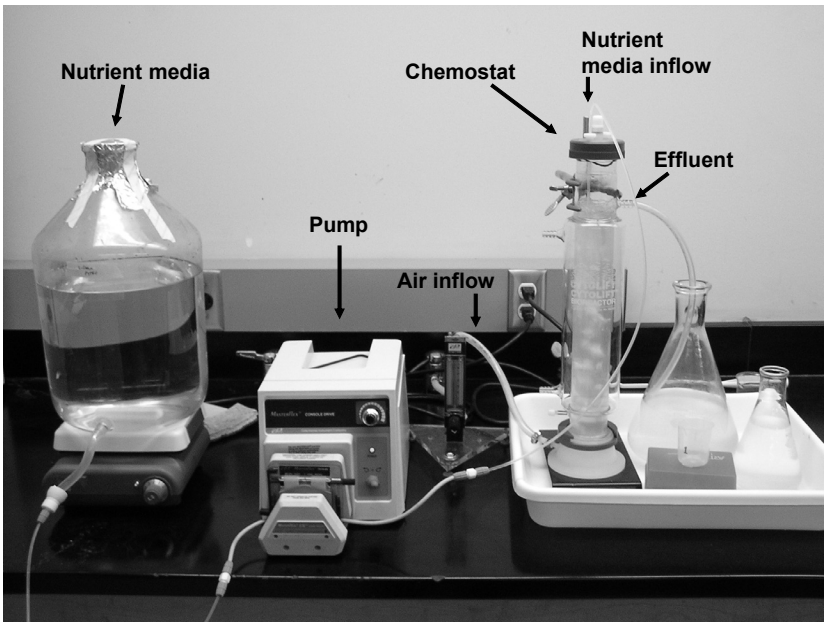

Figure 1. Illustration of a chemostat used in the study.

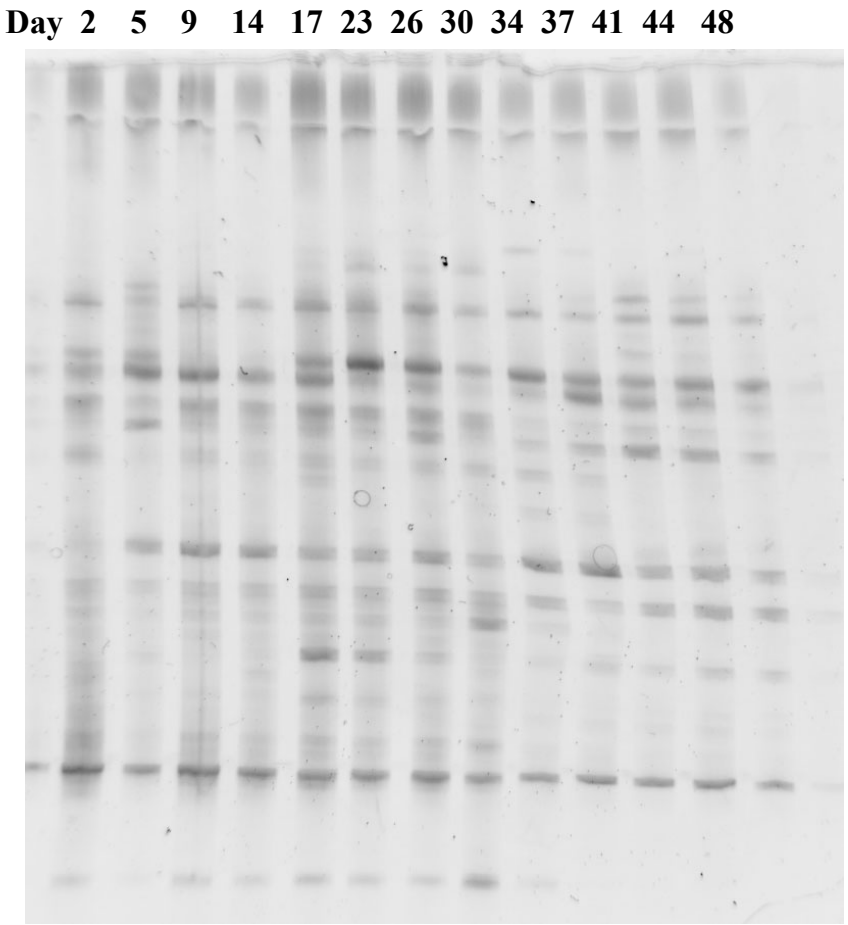

Figure 2. DGGE gel showing community fingerprint from selected days for Chemostat **2**.